Molecular Pharmacology

Loop diuretics are open-channel blockers of the cystic fibrosis transmembrane conductance regulator with distinct kinetics

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SUPPLEMENTAL INFORMATION

Supplemental Table 1 quantifies the kinetics of loop diuretic inhibition of CFTR at the single-channel level.

Supplemental Figure 1 shows overlays of the chemical structures of the four loop diuretics studied.

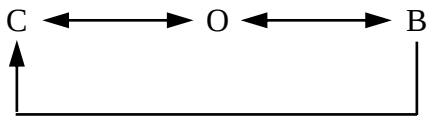
Supplemental Figure 2 investigates whether loop diuretics are allosteric blockers of CFTR by exploring whether changing the intracellular ATP concentration affects the efficacy of channel block.

Supplemental Figure 3 examines whether loop diuretic-blocked channels reopen first before closing on washout of loop diuretic, PKA and ATP from the intracellular solution.

SUPPLEMENTAL RESULTS

The closed ↔ open ↔ blocked kinetic scheme (Scheme I; eqn (2) in manuscript) predicts that CFTR must open before an open-channel blocker can access its binding site within the pore and prevent Cl^- permeation. Scheme I also predicts that CFTR is unable to close until after the blocker has dissociated from the pore. To provide further evidence that loop diuretics inhibit CFTR by occluding the pore, we investigated whether channels blocked by furosemide (250 μM)[1] would reopen first before closing on washing furosemide (250 μM), PKA (75 nM) and ATP (0.3 mM) from the intracellular solution. We reasoned that channel openings of normal amplitude should be observed on washout of furosemide, PKA and ATP prior to channel deactivation if furosemide inhibition of CFTR is described by Scheme I. Thus, if the current amplitude of individual CFTR Cl^- channels recovered completely prior to channel quiescence following washout of the drug, this would support Scheme I. However, if we observed no channel openings following drug washout, we would interpret the data to suggest that the channel closes directly from the blocked state (Scheme II). Using the simple kinetic model shown in eqn (2), we can represent Schemes I and II as follows:

Scheme I



Scheme II

Supplemental Figure 3A and B shows representative recordings from a furosemide washout experiment using a membrane patch that contained a single active CFTR Cl^- channel. Addition of furosemide (250 μM) to the intracellular solution decreased i and increased the

[1] We used an elevated concentration of furosemide to distinguish clearly channel block by furosemide in washout experiments.

frequency of flickery closures interrupting bursts of channel openings (Suppl Fig. 3B, trace 2). On washing furosemide (250 μM), PKA (75 nM) and ATP (0.3 mM) from the intracellular solution, i progressively returned to the control magnitude observed prior to the addition of drug (Suppl Fig. 3B, traces 3 and 4), before the channel became quiescent (Suppl Fig. 3B, trace 5). Supplemental Figure 3C demonstrates that identical results were observed in three other experiments.

Several different interpretations of the furosemide washout experiment are possible. First, CFTR closes via Scheme I on the dissociation of furosemide from the channel pore. Second, delays in the unbinding of ATP from the channel after the rapid dissociation of furosemide (Zeltwanger et al., 1999; Scott-Ward et al., 2004; present study) allow normal channels openings to be observed prior to channel deactivation. Third, CFTR closes via Scheme II, but then reopens prior to channel deactivation. When considered together with our other data, we suggest that the most plausible interpretation of the washout experiment is that the channel pore must "unblock" before CFTR closes. Thus, furosemide inhibition of CFTR is well described by the closed ↔ open ↔ blocked kinetic scheme. This argues that furosemide and other loop diuretics access their binding site on CFTR from the open channel configuration.

REFERENCES

Scott-Ward TS, Li H, Schmidt A, Cai Z, and Sheppard DN (2004) Direct block of the cystic fibrosis transmembrane conductance regulator Cl^- channel by niflumic acid. *Mol Membr Biol* **21:** 27-38.

Zeltwanger S, Wang F, Wang G-T, Gillis KD, and Hwang T-C (1999) Gating of cystic fibrosis transmembrane conductance regulator chloride channels by adenosine triphosphate hydrolysis: quantitative analysis of a cyclic gating scheme. *J Gen Physiol* **113:** 541-554.

SUPPLEMENTAL TABLE

Supplemental Table 1. Effects of furosemide and piretanide on the open- and closed-time constants of wild-type CFTR.

	[Furosemide] (μM)		[Piretanide] (μM)	
[Drug]	0	100	0	100
n	4	4	4	4
τ_{O1} (ms)	-----	-----	-----	6.0 ± 0.90
τ_{O2} (ms)	29.9 ± 4.80	2.40 ± 0.18	29.8 ± 5.30	19.3 ± 2.10
τ_{C1} (ms)	2.75 ± 0.13	3.93 ± 0.25	2.70 ± 0.20	1.60 ± 0.20
τ_{C2} (ms)	-----	-----	-----	6.40 ± 2.80
τ_{C3} (ms)	159.0 ± 20.4	148.8 ± 17.5	105.1 ± 13.5	130.8 ± 14.8
Area under curve τ_{O1}	-----	-----	-----	0.37 ± 0.07
Area under curve τ_{O2}	1	1	1	0.63 ± 0.07
Area under curve τ_{C1}	0.73 ± 0.04	0.95 ± 0.01	0.71 ± 0.05	0.23 ± 0.04
Area under curve τ_{C2}	-----	-----	-----	0.45 ± 0.06
Area under curve τ_{C3}	0.27 ± 0.04	0.05 ± 0.01	0.29 ± 0.05	0.32 ± 0.04
Events per minute	990 ± 170	3600 ± 330	865 ± 120	945 ± 113
Total time (s)	696	481	620	652

Supplemental Table 1. Effects of furosemide and piretanide on the open- and closed-time constants of wild-type CFTR. Open- and closed-time constants were measured at the indicated concentrations of furosemide and piretanide by the fitting of one-, two- or three-component exponential functions to open- and closed-time histograms. Area under curve indicates the proportion of the total dwell time distribution that corresponds to the different time constants. Events per minute represents the number of transitions between the open and closed states within one minute. The total time analyzed at each concentration of furosemide and piretanide is shown, and in each patch, approximately 5,000 events were analyzed per intervention. Values are means ± SEM of n observations. Measurements were made in the presence of the catalytic subunit of PKA (75 nM) and ATP (0.3 mM) in the intracellular solution. Voltage was –50 mV and there was a large Cl^- concentration gradient across the membrane ($[Cl^-]_{int}$ = 147 mM; $[Cl^-]_{ext}$ = 10 mM).

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Fig. 1. Three-dimensional chemical structures of loop diuretics. A, furosemide; B, bumetanide; C, xipamide and D, piretanide. Below the individual chemical structures, those of furosemide and xipamide (E) and of bumetanide and piretanide (F) are shown superimposed to highlight similarities and differences. Atoms are color-coded as follows: hydrogen, white; oxygen, red; carbon, green; nitrogen, blue and sulphur, yellow. Structures were rendered at a zoom factor of 8 Å using Pymol software (http://www.pymol.org).

Supplemental Fig. 2. CFTR inhibition by furosemide and piretanide is not relieved by elevation of the intracellular ATP concentration. A and B, the effects on CFTR Cl^- current of furosemide (100 μM) and piretanide (100 μM) when the intracellular solution contained either ATP (0.3 mM) or ATP (5 mM). Values represent the average CFTR Cl^- current recorded during the indicated interventions normalized to that measured under control conditions [ATP (0.3 mM)] at the start of the experiment. Data are means ± SEM (n = 4 for both furosemide and piretanide) acquired using excised inside-out membrane patches from BHK and C127 cells expressing wild-type human CFTR; the asterisks indicate values that are significantly different from the control value ($p < 0.05$). Comparison of the magnitude of CFTR inhibition at the different ATP concentrations reveals no difference in the potency of channel blockade (furosemide: 0.3 mM, 47 ± 7%; 5 mM, 42 ± 5%; n = 4 for both; $p = 0.29$; piretanide: 0.3 mM, 44 ± 6%; 5 mM, 35 ± 4%; n = 4 for both; $p = 0.15$).

Supplemental Fig. 3. Furosemide washout relieves channel block prior to deactivation of CFTR A, representative recording of a single CFTR Cl^- channel in an excised inside-out

membrane patch from a C127 cell expressing wild-type human CFTR during furosemide washout. Throughout the periods indicated by the filled bars ATP (1 mM), PKA (75 nM) and furosemide (Furo; 250 μM) were present in the intracellular solution. During the periods indicated by the dashed bars ATP, PKA and furosemide were washed from the intracellular solution. No data were collected while furosemide was perfused into the recording chamber. The dotted line indicates the closed channel state and downward deflections channel openings. The truncated upward and downward deflections of the trace represent noise artefacts caused by washing. Voltage was –50 mV and there was a large Cl^- concentration gradient across the membrane patch ($[Cl^-]_{internal}$ = 147 mM; $[Cl^-]_{external}$ = 10 mM). B, Expanded recordings corresponding to the sections marked 1 – 5 in A. C, values of i determined under the indicated conditions by cursor measurements using pCLAMP software. Data are means ± S.E.M. (n = 4); *, $p < 0.05$ vs. control. Other details as in A and Figure 2.